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Tumor promoter activity of estrogens and xenoestrogens is under investigation. Using the methylnitrosourea (MNU) treated rat, estrogens alone could not fully substitute for ovarian factors in mammary tumorigenesis. Estradiol or estrone replacement yielded 25% or 50% tumor incidence in ovariectomized rats. When progesterone was also administered alone it had no tumor promoting effect; treatment with progesterone plus estrone produced a 75% incidence. Thus, there is a synergy between progesterone and estrogen in mammary tumorigenesis.

The xenoestrogen, ß-hexachlorcyclohexane (ß-HCH) did not significantly promote tumorigenesis. Only 5 of 32 ovariectomized rats treated with ß-HCH had tumors 6 months after MNU injection; this was not different from tumor incidence in the control group. Dietary restriction did not affect the rate of tumorigenesis.

Both ß-HCH and another pesticide residue, o,p'-DDT induced growth of a human tumor xenograft in athymic mice. However this assay was inconsistent from between experiments and no conclusions could be drawn as to the effect of dietary restriction.

Low blood levels of xenoestrogen were associated with significant increases in esgtrogenic endpoints in ovariectomized mice. Levels averaging 42 ng/ml or 18 ng/ml for ß-HCH or o,p'-DDT, respectively produced increases in vaginal epithelial proliferation and induced hypertrophy of uterine epithelium. These levels are in the same order of magnitude as human blood levels.

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I. INTRODUCTION

Tumorigenesis is classically divided into two steps, initiation and promotion. Initiation is likely to be the result of some genotoxic insult. Tumor promotion depends on increased growth of the initiated cell and it may be the result of another genotoxic insult to growth regulatory machinery or it may derive from epigenetic stimuli that are normally involved in regulating growth of the original target tissue. Because of its role in mammary gland development, estrogen has long been suspected to behave as a tumor promoter of mammary cancer. This notion is supported by the fact that mammary tumor growth may be inhibited by antiestrogens. It follows that xenoestrogens could supply tumor promoter activity to initiated mammary cells. The studies devised under this grant are designed to examine the tumor promoter activity of two xenoestrogens, o,p'-DDT and ß-hexachlorocyclohexane (ß-HCH), to define the role of fat stores of these compounds in mammary tumorigenesis, and to determine the molecular mechanism of estrogenic action of o,p'-DDT and ß-HCH.

The body of this report will be divided into sections according to research models studied: 1) the rat methylnitrososurea (MNU)-induced mammary tumor model; 2) human mammary tumor xenograft in athymic mice; 3) the ovariectomized mouse uterine and vaginal epithelial bioassays. The Results and Discussion subsection of each report will deal with the scientific significance of the observations made to date, while a separate section will be allotted to the discussion of the relevance to the original Statement of Work.

II. BODY OF REPORT

1. MNU-INDUCED MAMMARY TUMORS IN THE RAT

A. BACKGROUND & INTRODUCTION

MNU induces estrogen-dependent mammary tumors in the rat and this has become a standard model system for testing tumor growth inhibiting activity of potential estrogen antagonists. Since injected MNU is fully oxidized and disappears from the animal's system within a matter of less than one hour (1-3), it must produce its tumor initiating effects within minutes of administration. Fully mature, young rats (age, 49-56 days) have been shown to respond optimally to the tumorigenic effect of MNU. Estrogen-dependence of MNU-induced tumors is demonstrable by the following observations: ovariectomy or antiestrogen treatment performed after MNU initiation blocks tumor development; ovariectomy leads to tumor stasis or shrinkage and estrogen replacement will cause resumption of tumor growth. In other model systems the xenoestrogens, o,p'-DDT and ß-HCH have been shown to support growth of experimental mammary tumors and/or human breast cancer cells in culture (4, 5). These observations suggest that estrogen is the ovarian factor that is required for tumor progression and that xenoestrogens can substitute for the natural hormone in this regard. However, the tumor promoter activity of estrogens has not been tested directly in the standard MNU rat model.

A study was designed to address the question of whether estrogen behaves as a tumor promoter in the MNU-initiated rat model. There is only one prior report in which MNU-treated animals were ovariectomized before detectable tumors were formed and then treated with estrogen to promote tumorigenesis (6). That study was performed in an unorthodox protocol in which neonatal (2-day-old) rats were treated with MNU, followed by tamoxifen treatment until 30 days of age, at which time animals were ovariectomized, and then estrone treatment was begun at 3.5 months of age. In this protocol, 50% (6/12) estrogen reconstituted animals developed tumors compared to rates of 80% (35/44) in ovary-intact control animals and 4% (1/27) in ovariectomized, untreated, MNU-initiated rats. Thus, from this single study, it would appear that estrogen can act as tumor promoter in an MNU-initiated mammary gland and therefore estrogen-replacement in ovariectomized MNU-initiated animals should serve as the positive control for the xenoestrogen studies.

In our study, animals (49-56 days old) were ovariectomized at the time of MNU treatment and either supplied with a continuous release estrogen capsule or received no further treatment. The notion that estrogen serves a tumor promoter in the mammary gland was directly tested in this manner. In addition, this model was used to test of whether xenoestrogen treatment of ovariectomized rats would be sufficient to insure tumor progression. The experiments completed to date tested the tumor promoter activity of estradiol and estrone, the latter being a weaker estrogen that predominates in postmenopausal women and which may be more comparable to the weakly estrogenic xenobiotics. Our earlier data indicated that animals supplemented with estradiol had a tumor incidence of only 24% while those treated with estrone had a 55% tumor incidence. Thus, there may be a fundamental difference in the tumor promoter activitys of these two estrogens, and neither alone is sufficient to reproduce the effect of

the ovary. Further experiments were performed in which estrone and progesterone were administered in an attempt to achieve full tumor promoter activity.

Others had reported that high doses of estrogen, with or without progesterone, could actually protect against MNU-induced tumorigenesis (7). It may be that the estrogen treatments applied in our experiments are also exerting a degree of protection, and that the final tumor incidence is a matter of balance between tumor promoter and anti-tumor action of the estrogen. Thus, groups of MNU-treated, ovary-intact animals were treated with estradiol or estrone.

B. METHODS

Sprague-Dawley rats (49-56 days old) were ovariectomized and treated with 50 mg/kg MNU (Sigma Chemical Co., St. Louis, MO) by tail vein injection while still under anesthesia (Ketamine). At the time of ovariectomy, a treatment capsule was implanted subcutaneously in each animal. The capsules were made from Silastic tubing (Konigsberg Instruments, Pasadena, CA, 0.062 in. I.D. X 0.125 in. O.D. X 1.4 cm length), sealed at each end with Silastic cement and containing 26-30 mg of crystalline estrone, or in the case of the negative control group, the implants remained empty. The effect of estrone was further examined by reducing its dose; capsules were made with estrone at a 1:100 dilution by mixing it with crystalline cholesterol. Groups of animals were also treated with progesterone alone or in combination with estrone (at full strength or at 1:100); the progesterone was delivered in a single capsule made as with estrone. As a positive control, animals were left intact and implanted with empty Silastic capsules. In two other groups, animals were left intact, treated with MNU and had a single capsule of estradiol or estrone implanted at the time of MNU treatment. Beginning at 4 weeks after treatment and continuing through 26 weeks, animals were palpated on a weekly basis to determine the time at which tumors appeared in each animal.

The effects of ß-HCH were tested in two additional groups of animals. Each of these animals received 4 implants of ß-HCH and one group was placed on periodic dietary restriction throughout the 6 month observation period. Dietary restriction consisted of giving animals 40% of their usual daily intake ffor 1 week out of every three weeks.

At the end of the experiment (26 weeks), animals were killed by cervical dislocation. Whole body, pituitary, and uterine weights were recorded; tumors were collected and processed for histologic examination. Each animal was skinned to allow visualization of the mammary glands to determine if small tumors had escaped detection during palpation. Mammary tumors were dissected out and processed for histologic examination.

Mammary glands were also examined by the whole mount technique as described previously (last annual report). In addition to the experimental animals described above, groups of animals were ovariectomized and treated with hormone as above, without MNU injection. Animals in these treatments were killed at 24 h, 1 week, or 1 month for collection of blood. The mammary glands of animals killed at 1 month were prepared for whole mount observation. Blood serum was sent to Dr. Clinton Grubbs, University of Alabama for analysis of estradiol and progesterone levels.

Tumor incidence was analyzed by Chi-square analysis. All other parameters were analyzed by ANOVA, followed by Fisher's PLSD to test for differences between individual treatment groups.

C. Results & Discussion

We previously reported that tumor incidences in the ovariectomized animals were 55% (6/11) of estrone-treated and 24% (5/21) of estradiol-treated animals. The ovariectomized, MNU-treated controls produced no tumors, while 100% of the intact, MNU-treated controls had tumors. Furthermore, uterine and pituitary weights indicated that the estrogen treatments remained effective at the end of the 8 month experiment.

Since maximal tumor incidence was reached by 6 months in our earlier experiment, we analyzed this parameter at 6 months in the present set of animals. Estrone produced a 45% incidence and this was increased to 75% in the estrone plus progesterone group (Figure 1). The diluted estrone was ineffective with or without the addition of progesterone.

Examination of mammary glands by whole mount preparation showed that one month of hormone treatment induced a growth response in the tissue (Fig. 2). Mammary glands of intact control animals had an abundance of end buds decorating the ducts, while the ducts of ovariectomized animals were straight and had very few end buds. Treatment with estrone or estradiol induced growth of the mammary tree such that the ducts were decorated with end buds, but not to the extent of the normal gland. Progesterone alone had no effect on mammary gland growth. The combination of progesterone and estrone produced and even greater growth response.

Estradiol and progesterone blood levels during this initial treatment were analyzed as in the previous report. Blood estrogen levels were analyzed using a radioimmunoassay for estradiol; in the manner used, the assay measures both estrone and estradiol. In estrone-treated animals, estrogen levels reached a peak of 60-80 pg/ml during the first week, returning to 40 pg/ml by one month. We had previously reported that the estrogen concentrations were 18-24 pg/ml after 8 months of treatment (previous report).

In intact animals, both estradiol and estrone exerted a protective effect (Fig. 3). In this respect estradiol was the more efficient hormone, reducing the tumor incidence by 67% compared to a 44% reduction from estrone.

Clearly, estrogens produced a tumor promoter effect in this MNU model system. However, in light of the obvious growth response that the estrogen treatments produced, the lack of a full tumor promoter activity seemingly poses a paradox. Earlier studies have shown that large doses of estrogen will protect against tumor formation in MNU-treated rats (7); furthermore, it was recently suggested by Guzman et al (8) that this effect is dependent upon a sufficient dose of estradiol to attain pregnancy levels of hormone in the blood serum (ca. 150 pg/ml) for only one week. It may be that the low incidence of tumors in our estradiol-treated group can be attributed to this protective effect. We had estimated that our Silastic capsules release approximately 1 μg estradiol/day (9). During the first week of treatment blood levels of estrogen were as

high as 80 pg/ml and at the end of 40 weeks, serum estrogen was 25-30 pg/ml in estradiol-treated animals and 18-24 pg/ml in estrone-treated animals. The data reported by Guzman et al (8) not only showed that the highest dose of estradiol used in their experiments was effective at reducing tumor incidence in MNU-treated rats but a dose of one-tenth that which produced the pregnancy levels of estradiol in blood was similarly effective, and a further 10-fold reduction of dose still produced some protective effect. We too found that low estradiol levels can exert protective effects in the MNU-treated rat. Thus, estrogen can have both a protective effect and a promoting effect on mammary gland tumorigenesis. The biphasic nature of the hormone's effect in this regard is not atypical of hormone action in general but it requires further investigation to determine the boundary between these two opposing responses.

ß-HCH was ineffective as a tumor promoter, with or without dietary restriction. Only 5 of the 32 animals in the two ß-HCH groups (± dietary restriction) had tumors, this was not significantly different from the ovariectomized control group rate of1 out of 30.

2. MOUSE MODEL – MCF-7 XENOGRAFTS

A. BACKGROUND & INTRODUCTION

The xenograft of human tumor cells in athymic mice has become another standard model for testing the tumor growth promoting or inhibiting effects of a test compound. We had shown earlier that ß-HCH stimulated growth of tumors produced from the human breast cancer cell line, MCF-7 (5). In that study, host animals were treated with a single Silastic capsule containing either ß-HCH or estradiol; tumor growth was essentially equivalent for both treatments over a 16 day period.

As of our last Annual Report, these observations could not be reproduced. The tumors produced exhibited a much slower growth rate than earlier and they were not at all responsive to xenoestrogen. We have made two additional attempts to repeat that earlier observation.

B. METHODS

MCF-7 Xenograft Experiment

Two separate experiments were performed. In each case host animals (5-6 week old, athymic, female Balb/c mice) were implanted with tumor that was derived from tumors passaged in athymic mice. The original tumor was produced by injection 10^7 MCF-7 cells in Matrigel subcutaneously, as described in the previous report. Once the resultant tumor reached 1.5 cm diameter, the host was killed and the tumor was excised. Tumor tissue was minced and passed through an 40 guage stainless steel mesh (Cellector) and suspended in culture medium (DMEM). The tumor tissue was washed by allowing the fragments to settle out of suspension, removing and replacing the medium. A final suspension was made to 50% (vol/vol) and this was used for injection. Each host animal received two subcutaneous injections (100 μl) of tumor slurry through an 18 guage needle.

In the first experiment, a single Silastic capsule containing estradiol was inserted subcutaneously at the time of tumor implantation. After 5 weeks of tumor growth, the estradiol capsules were removed from 28 animals and 16 of these were treated by inserting 4 capsules of ß-HCH into each animal, the other 12 animals were left

untreated. There were 6 animals in which the original E2 capsule remained; these served as positive controls. After one additional week, 8 of the ß-HCH-treated animals and 6 of the untreated animals were placed on periodic food restriction; they received 50% of the normal food intake for 3 days of each week. Tumors were measured with calipers and tumor volumes were determined, as described earlier.

In experiment no. 2, the tumor used was derived from a tumor that grew slowly in an intact athymic host without added E2. It was hoped that this tumor would prove more sensitive to weak estrogens. This tumor was transplanted to experimental host animals and each host was given three daily injections of estradiol (10 μg/kg/day) to insure tumor take and initial growth. Three weeks after the last E2 injection, groups of animals received a single capsule of E2, 4 capsules of o,p'-DDT, or 4 capsules of ß-HCH and tumor growth was followed on a weekly basis; animals that received no further treatment after the initial injections served as negative controls. Tumor volume was measured on a weekly basis.

C. Results and Discussion

As of our last Annual Report, the original observations of xenoestrogen-induced tumor growth could not be reproduced. The tumors produced exhibited a much slower growth rate than earlier and they were not at all responsive to xenoestrogen. Further attempts to repeat this original observation have produced mixed results. Figures 4 and 5 show the growth responses in two separate experiments. In the first experiment, tumor was initially stimulated by implantation of an estradiol capsule. Experimental treatments began with removal of the E2 capsule after 5 weeks of growth; at this time the average tumor volume was equal to 570 mm^3. In this experiment, ß-HCH did not substitue for E2 (Fig. 4); furthermore, fasting had no effect on tumor growth.

In the second experiment the implanted tumor tissue came from a tumor that had grown in an intact female host without steroid supplementation. In this case the tumor volume at the beginning of the experimental treatments was much smaller, with an average of 45 mm^3. These tumors grew under estradiol and o,p'-DDT stimulation; ß-HCH produced only a small growth effect (Fig. 5). This is in contrast to the original experiments which indicated that ß-HCH was as active as estradiol in this growth assay (5).

The reasons for the inconsistencies in this tumor growth assay are unknown. Perhaps there has been a subtle change in the character of the MCF-7 cells used. Unfortunately, due to an accident in the lab we have lost all of the early passages of the MCF-7 cell stock. Clearly, the original tumors grew at a much faster rate and they continued growth for several days without estradiol supplementation (5). Also, the size of the tumor at the initiation of experimental treatment may be very important; here, the very small tumors responded to o,p'-DDT and ß-HCH while the tumors that were large at the outset did not respond.

3. OVARIECTOMIZED MOUSE MODEL – UTERINE & VAGINAL EPITHELIAL BIOASSAYS

A. BACKGROUND & INTRODUCTION

As indicated in the last Annual Report, blood and tissue levels attained following implantation of Silastic capsules containing either ß-HCH or o,p'-DDT were related to the biological response in ovariectomized mice. In our earlier report it was shown that all doses used produced significant estrogenic effects. Thus, the treatment regime needed to be extended to lower doses to establish the lowest blood levels which would be associated with a significant increase in estrogenic endpoints. This study is completed and has yielded surprising dose-response information.

B. METHODS

Dose-Response of op-DDT and ß-HCH administered to mice.

Animal treatment: Silastic capsules were made as described above with the exception that the length of capsule containing crystalline compound was 0.8 cm so that each capsule contained 18-22 mg of material. To achieve lower doses, the crystalline test compound, o,p'-DDT or ß-HCH, were diluted by mixing with crystalline cholesterol. Mixtures were made to yield 1/2, 1/4, or 1/8 of the dose. Adult ICR mice were ovariectomized and 3 weeks later groups of five animals received 1 treatment capsule containing either undiluted or diluted test compound, some animals received 2 capsules at 1/2 dilution, and others received 2, or 4 capsules of undiluted treatment compound. Thus, this regimen covered a 32-fold range of doses. A positive control group consisted of animals that received a Silastic capsule containing estrone (Sigma) and negative control animals were treated with an empty Silastic capsule. After 1 week of treatment, animals were anesthetized and exsanguinated by heart puncture. The uterus and vagina of each animal was processed for histomorphometric determination of the estrogenic effects; blood serum and intraperitoneal fat samples were analyzed for o.p'-DDT or ß-HCH content by gas chromatography.

Histomorphometrics and Blood Levels of o,p'-DDT or ß-HCH: As described in the previous report, the height of the uterine epithelium and thickness of the vaginal epithelium were used as endpoints of estrogenic activity. These parameters were then correlated to blood levels of o,p'-DDT or ß-HCH as measured by gas chromatography (see methods in previous report).

C. RESULTS AND DISCUSSION

Blood and fat levels of ß-HCH or o,p'-DDT correlated linearly (Fig. 6). It is apparent from these linear increases that saturation of the tissues has not been reached, even at the highest dose applied. Macholz et al (10) showed that feeding rats food containing either 600 or 3000 ppm ß-HCH produced concentrations in the fat of 250 μg/g or 210 μg/g, respectively, at 30 days, indicating that the fat was saturated. The highest level achieved in our study was 55 μg ß-HCH/g fat tissue at the end of one week of treatment. In an earlier study, Dale et al (11) reported that feeding rats 200 ppm of DDT for 90 days produced fat levels of 729-2206 μg/g in female rats. In our study, after one week with 4 treatment capsules the fat levels of op-DDT were 56-99 μg/g.

The lowest dose of compound used in this mouse study produced blood and fat levels that were similar to levels recorded in humans with no history of excess exposure. The

lowest dose of o,p'-DDT in our study produce blood levels of 4.4 ± 0.61 ng/ml and fat levels of 265 ± 19 ng/g. Human blood levels of all isomers of DDT and their metabolites are generally 5-30 ng/ml (12). Although the o,p'-isomer is only a very small proportion of total DDT isomers contaminating the environment, a group of Israeli men were found to have blood levels of o,p'-DDT as high as 32 ng/ml (12). In the present mouse study, the lowest dose of ß-HCH produced blood levels of 5.0 ±0.97 ng/ml. Human blood levels of ß-HCH are generally 0-5 ng/ml but have averaged as high as 23 ng/ml in non-exposed populations or as high as 240 ±140 ng/ml in occupationally exposed populations (12).

Uterine epithelium exhibits both hypertrophic and hyperplastic responses to estrogen stimulation. The hyperplastic response is apparent from the extremely crowded character of the epithelium; although it is a simple type of epithelium, under estrogen stimulation it takes on the appearance of a stratified layer of cells, i.e. it becomes pseudostratified. The hypertrophic response of the epithelial cell is apparent from its increase in cell height. Upon ovariectomy, the columnar epithelium becomes cuboidal with an average height of approximately 7.7 μm; stimulation with estrone increased the epithelial height to 25 μm (Table 1). o,p'-DDT increased uterine epithelial height to 26 μm in a dose dependent fashion . The uterine response to ß-HCH was quite different; a single treatment capsule produced a significant increase in epithelial cell height, to 12 μm, but there was no further increase with increasing dose.

Vaginal epithelium undergoes a dramatic increase in cell proliferation in response to estrogenic stimulation. With full stimulation from a strong estrogen the superficial layer of cells loose their nuclei and organelles to become a flattened layer of keratin; continuous stimulation produces multiple laminations within this keratinized superficial layer. In the ovariectomized control animals the epithelium consisted of a very low layer of squamous cells that was one to two cells thick, approximately 12 μm. Estrone-treated animals had a vaginal epithelium that was multilayered with a fully keratinized surface layer; the epithelial thickness, below the keratinized layer, was 78-97 μm. Thickness of the vaginal epithelium was increased in a dose-dependent manner after treatment with either o,p'-DDT or ß-HCH (Table 1). The epithelium was keratinized in animals treated with the 3 highest doses of o,p'-DDT. In ß-HCH-treated animals, only two of the five animals with four treatment capsules exhibited a keratinized epithelium and none of the others had a keratinized surface layer. Thus there appears to be both a quantitative and a qualitative difference between the estrogenic characters of ß-HCH and o,p'-DDT.

The object of these dose-response studies was to determine the lowest dose of compound that would produce an estrogenic effect and to correlate tissue levels against the estrogenic effect. This would aid in completion of our present studies in that knowing the effective blood and tissue levels will help in the interpretation of the data on tumor growth in both the rat model (above) or in the mouse xenograft model. It would also be useful for comparing known human blood and tissue levels against putative estrogenic effects. We found that blood levels of 18 ± 2.3 ng/ml o,p'-DDT or 42 ± 3.6 ng/ml ß-HCH were associated with statistically significant increases in estrogenic endpoints. Thus, blood levels that are within the same order of magnitude as those found in humans have proved to be estrogenic.

4. RELEVANCE TO STATEMENT OF WORK

YEAR 2, ORIGINAL SOW

Aim1: Complete experiment on tumor promoter effects under continuous tratment; process tissues (brast tumors, uteri, vaginas); analyze data. Begin tumor promter experiment with animals that are fed or diet-restricted. Collect and analyze blood and tissue samples form this experiment.

Aim 2: Complete xenograft experiments with fed vs. fasted hosts. Collect and analyze blood samples.

Aim 3: Begin and complete transfection experiments.

PROGRESS TO DATE:

Aim 1: Most of the work to date on this, the main aim of the project, has centered around a determination of the effectiveness of natural estrogens to promote mammary tumorigenesis. It was found that estradiol or estrone alone promote tumors only partially, i.e. they produce tumor incidences of about 25% and 50%, respectively, in ovariectomized animals. When progesterone was added, estrone induced tumors in 75% of the animals; progesterone alone had no effect. Thus, there is a synergistic tumor promoter activity of progesterone and estrone and this synergy is required for full tumorigenic activity.

A single experiment has been performed in which the xenoestrogen, ß-HCH, was administered to ovariectomized, MNU-treated animals. In addition, this experiment included a group of ß-HCH-treated animals that were placed on periodic food restriction. There was no indication that ß-HCH acted as a tumor promoter on its own. Further work is required to determine if progesterone plus xenoestrogen will promote tumorigenesis.

Aim 2: After 3 experiments, this tumor growth assay has behaved inconsistently. The rate of tumor growth in all 3 experiments did not approach that of our original, published observations. ß-HCH was only slightly stimulatory in one of the three experiments and o,p'-DDT was fully stimulatory in one of two experiments in which it was tested. The only experiment in which fasting was added as a variable, the xenoestrogen under test, ß-HCH was ineffective, with or without food restriction. Thus, there are no solid conclusions to be drawn from these experiments.

Aim 3: Work on this aim was completed.

III. CONCLUSIONS

Although the standard MNU-initiated rat model would seem to be the ideal model system for doing so, the tumor promoter effects of ovarian hormones has never been established in this system. Our studies have shown that stimulation of mammary gland growth by estradiol or estrone is not in itself sufficient to promote tumorigenesis to same extent as the intact ovary. In addition, it appears that there is a biphasic response to estrogens: they are able to both inhibit and promote tumorigenesis. Although it was considered controversial (13), it may be that progesterone supplied by the ovary is also essential for full tumor promoter activity. This was tested in the ovariectomized rat. Progesterone alone had no promoter effect but it synergistically enhanced estrone's tumor promoter activity. This supports the recent epidemiological findings that combined hormone replacement therapy, estrogen plus progestin, is associated with an increased risk for breast cancer (14, 15).

The studies on estrogenic effects of op-DDT and ß-HCH in the mouse reproductive tract have produced some startling results. This is the first study in which blood levels of test compound were correlated to estrogenic response. In past reports xenoestrogens were administered in very large doses (100-500 mg/kg BW) and acute estrogen responses were monitored (4, 16-19). The impression from such studies was that xenoestrogens, being very weakly estrogenic by a number of *in vitro* assays (19-22), required the very large doses of compound to have an effect *in vivo*. In our study the compounds were delivered continuously in low doses. The blood levels attained by the highest doses in the mice were well above those which are found in the general population of people. However, the lowest dose that induced significant biological effects in the mice produced blood levels that were within the same order of magnitude of blood levels found in some non-exposed humans (12). Thus, under the condition of a continuous low level of exposure, estrogenic effects can be expected from levels of xenoestrogen commonly encountered in humans.

The MCF-7 xenograft studies have been inconsistent and no conclusions can be drawn at this date. The tumors formed from the cells now available do not seem to have the same growth characteristics as those of our earlier study. And xenoestrogens have inconsistently stimulated tumor growth. In the single experiment in which food restriction was applied, the xenoestrogen was ineffective with or without dietary restriction.

The year 1 studies on the estrogen receptor domains which participate in estrogen and xenoestrogen transcriptional activation have given us some clues as to how compounds can produce qualitatively and quantitatively disparate effects in the same tissue. Compounds such as BPA, octylphenol, and ß-HCH are fully effective compared to estradiol when the wild-type receptor is present; o,p'-DDT produces a super-activation of the reporter gene. Deletion of either AF-1 or AF-2 results in a loss of relative efficacy of the xenoestrogens. Thus, they appear to require a synergy between the two activation function domains. Recent observations by Nishikawa (23) suggest that the different compounds are likely to produce different receptor–coactivator interactions. The fact that o,p'-DDT consistently produced a greater response than estradiol suggests that it may enhance binding of coactivator to receptor. Such differences in molecular

activity of compounds should be considered when attempting to assess their potential bioactivity.

V. REFERENCES

1. Cox R. Differences in the removal of N-methyl-N-nitrosourea-methylated products in DNase I-sensitive and -resistant regions of rat brain DNA. Cancer Research 1979; 39, 2675-2678.
2. Wilander E, Tjalve H. Uptake of labelled-n-nitrosomethylurea in the pancreatic islets. Virchows Archiv A, Path Anat Histol 1975; 367, 27-33.
3. Woolley PV, Pinsky SD, Yerino P. Distribution and binding of the carcinogens 1-methyl-1-nitrosourea and 1-methyl-3-nitro-1-nitrosoguanidine in the guinea pig after oral administration. Carcinogenesis 1982; 3, 1443-1447.
4. Robison AK, Sirbasku DA, Stancel GM. DDT supports the growth of an estorgen-responsive tumor. Tocicology Lttrs 1985; 27, 109-113.
5. Steinmetz R, Young PC, Caperell-Grant A, et al. Novel estrogenic action of the pesticide residue beta-hexachlorocyclohexane in human breast cancer cells. Cancer Research 1996; 56, 5403-5409.
6. Kumar R, Sukumar S, Barbacid M. Activation of ras oncogenes preceding the onset of neoplasia. Science 1990; 248, 11-1-1104.
7. Grubbs CJ, Farnell DR, Hill DL, McDonough KC. Chemoprevention of N-nitroso-N-methylurea-induced mammary cancers by pretreatment with 17 beta-estradiol and progesterone. J Natl Can Inst 1985; 74, 927-931.
8. Guzman RC, Yang J, Rajkumar L, Thordarson G, Chen X, Nandi S. Hormonal prevention of breast cancer: mimicking the protective effect of pregnancy. Proc Natl Acad Sci U S A 1999; 96, 2520-2525.
9. Steinmetz R, Mitchner NA, Grant A, Allen DL, Bigsby RM, Ben-Jonathan N. The xenoestrogen bisphenol A induces growth, differentiation, and c-fos gene expression in the female reproductive tract. Endocrinology 1998; 139, 2741-2747.
10. Macholz RM, Bleyl DW, Klepel H, et al. [Comparison of the distribution and toxicity of alpha-, beta-, and gamma-hexachlorocyclohexane (HCH) following 30 days of administration to rats]. Nahrung 1986; 30, 701-708.
11. Dale WE, Gaines TB, Hayes WJJ. Storage and excretion of DDT in starved rats. Toxicol Appl Pharmacol 1962; 4, 89-.
12. Toppari J, Larsen JC, Christiansen P, et al. Male reproductive health and envrionmental xenoestrogens. Environ Health Perspect 1996; 104, 741-803.
13. Soderqvist G. Effects of sex steroids on proliferation in normal mammary tissue. Ann Med 1998; 30, 511-524.
14. Schairer C, Lubin J, Troisi R, Sturgeon S, Brinton L, Hoover R. Menopausal estrogen and estrogen-progestin replacement therapy and breast cancer risk. JAMA 2000; 283, 485-491.
15. Ross RK, Paganini-Hill A, Wan PC, Pike MC. Effect of Hormone Replacement Therapy on Breast Cancer Risk: Estrogen Versus Estrogen Plus Progestin. J Natl Cancer Inst 2000; 92, 328-332.
16. Cummings AM. Replacement of estrogen by methoxychlor in the artificially-induced decidual cell response in the rat. Life Sci 1993; 52, 347-352.
17. Johnson DC, Kogo H, Sen M, Dey SK. Multiple estrogenic action of o,p'-DDT: Initiation and maintenance of pregnancy in the rat. Toxicology 1988; 53, 79-87.
18. Loeber JG, van Velsen FL. Uterotrophic effect of ß-HCH, a food chain contaminant. Food Add Contam 1984; 1, 63-66.

19. Nelson JA. Effects of dichlorodiphenyltrichloroethane (DDT) analogs and polychlorinated bihphenyl (PCB) mixtures on 17ß-[3H]estradiol binding to rat uterine receptor. Biochem Pharmacol 1974; 23, 447-451.
20. Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FO. The E-screen assay as a tool to identify estrogens: An update on estogenic environmental pollutants. Environ Health Perspect 1995; 103 , 113-122.
21. Klotz DM, Beckman BS, Hill SM, McLachlan JA, Walters MR, Arnold SF. Identification of environmental chemicals with estrogenic activity using a combination of in vitro assays. Environ Health Perspect 1996; 104, 1084-1089.
22. Gaido KW, Leonard LS, Lovell S, et al. Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay. Toxicol Appl Pharmacol 1997; 143, 205-212.
23. Nishikawa J, Saito K, Goto J, Dakeyama F, Matsuo M, Nishihara T. New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. Toxicol Appl Pharmacol 1999; 154, 76-83.

Table 1. Blood Concentrations and Biological Endpoints. Mean values of blood serum concentrations, uterine epithelial cell height (UEH) and vaginal epithelial thickness (VET) were determined for each treatment/dosage group. For *o,p'*-DDT and β-HCH, dosage groups are listed as A-G for treatments with a single diluted capsule (1/8, 1/4, 1/2), a single undiluted capsule (1) or multiple capsules (2X 1/2, 2, 4). Values are presented as means ± SEM; numbers in parentheses following the mean refer to the number of samples analyzed. *, $p < 0.05$ or **, $p < 0.005$ compared to control; nd, not determined

Treatment group	Blood Conc. (ng/mL)	UEH (μm)	VET (μm)
Control	0	7.74 ± 0.32 (5)	11.7 ± 0.94 (5)
Estrone	nd	25.5 ± 3.5 (5)**	79.6 ± 6.6 (5)**
o,p'-DDT			
A (1/8)	4.4 ± 0.61 (4)	7.88 ± 0.62 (4)	14.3 ± 2.5 (4)
B (1/4)	5.2 ± 0.91 (3)	8.93 ± 0.22 (3)	14.7 ± 0.87 (3)
C (1/2)	23 ± 13 (2)	9.15 ± 0.66 (3)	20.0 ± 1.7 (4)*
D (2X1/2)	18 ± 2.3 (3)	12.0 ± 0.95 (5)**	39.7 ± 7.0 (5)**
E (1)	190 ± 40 (4)	11.4 ± 1.6 (4)*	63.9 ± 10 (4)**
F (2)	260 ± 26 (4)	15.7 ± 1.9 (4)**	85.3 ± 21 (4)**
G (4)	620 ± 150 (4)	26.0 ± 1.7 (4)**	114 ± 8.4 (4)**
β-HCH			
A (1/8)	5.0 ± 0.97 (3)	8.40 ± 0.61 (4)	12.1 ± 1.7 (4)
B (1/4)	12 ± 3.4 (3)	7.18 ± 0.75 (4)	15.1 ± 1.1 (4)
C (1/2)	22 ± 2.5 (3)	9.03 ± 1.2 (5)	17.6 ± 4.2 (5)
D (2X1/2)	42 ± 3.6 (3)	9.97 ± 1.0 (4)	21.9 ± 2.7 (5)**
E (1)	66 ± 3.9 (3)	12.1 ± 0.61 (4)**	38.3 ± 6.1 (4)**
F (2)	170 ± 29 (4)	10.8 ± 0.71 (5)*	38.9 ± 2.9 (5)**
G (4)	300 ± 49 (4)	11.3 ± 0.82 (5)*	58.1 ± 5.2 (5)**

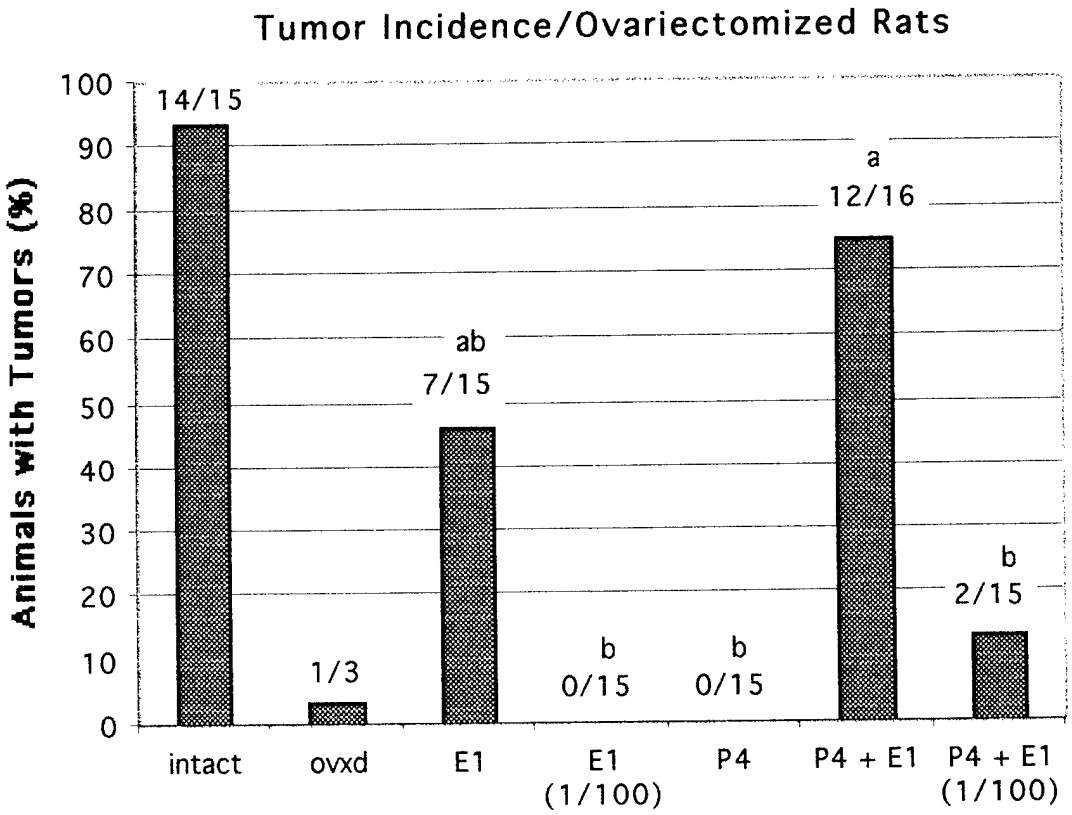

Figure 1. Tumor Incidence in MNU-Treated, Ovariectomized Rats. Rats were ovariectomized, injected with MNU and a Silastic treatment capsule was inserted subcutaneously. Treatment capsules contained crystalline estrone (E1), crystalline estrone at a 1/100 dilution (E1 1/100), or progesterone P4; in one group two capsules containing progesterone or estrone were inserted(P4+E1). Controls included ovary intact animals (intact) and ovariectomized animals (ovxd). The tumor incidence was recorded at 6 months and analyzed by Chi-square. a, $p < 0.05$ vs ovxd; b, $p < 0.05$ vs. intact.

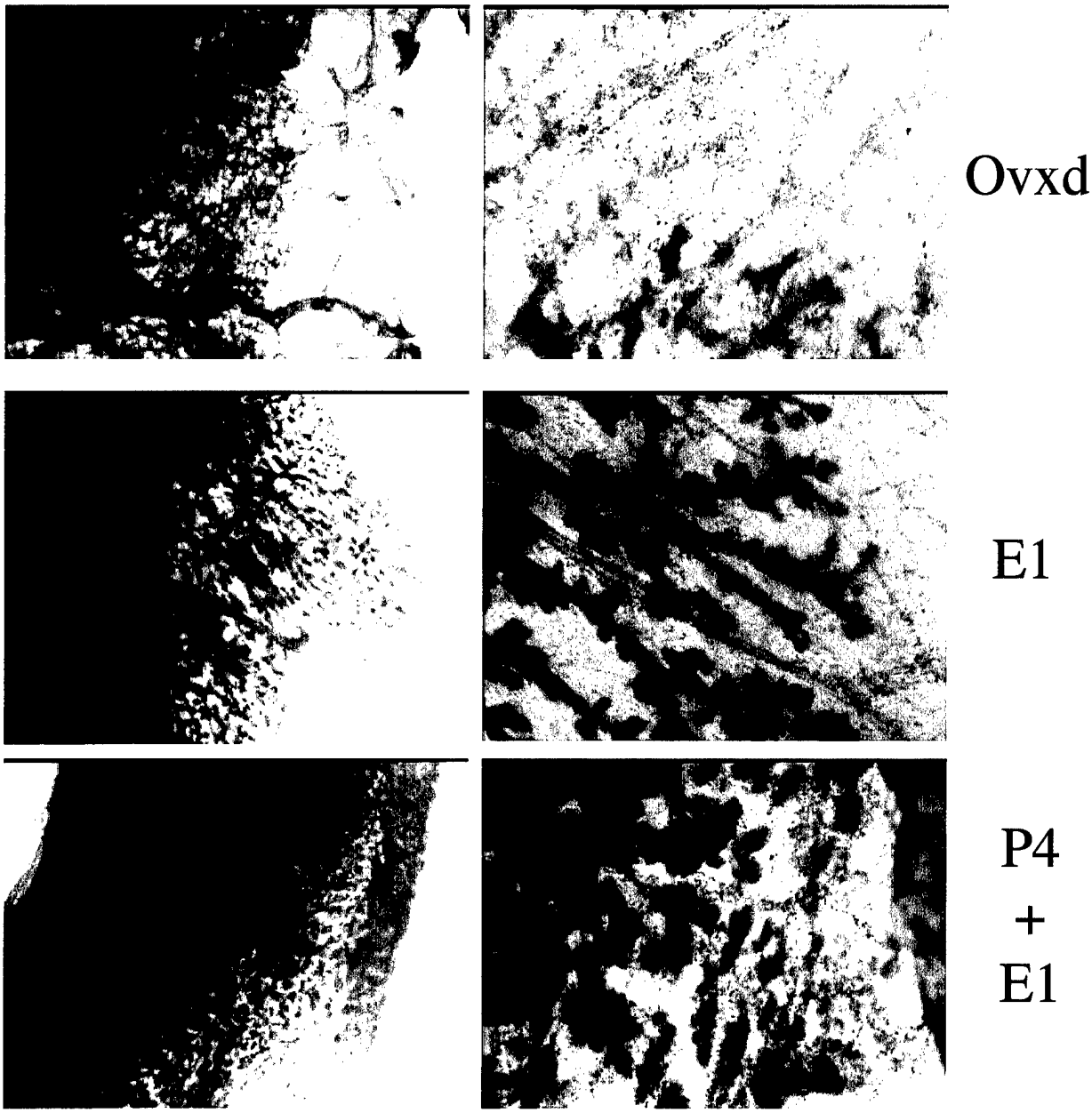

Figure 2. Mammary Whole Mounts. Ovariectomized rats were treated for 1 month with an empty Silastic capsule (ovxd), an estrone capsule (E1), or a progesterone capsule and an estrone capsule (P4 + E1). Whole mount preparations were viewed at low (left) and high (right) power on a dissecting microscope.

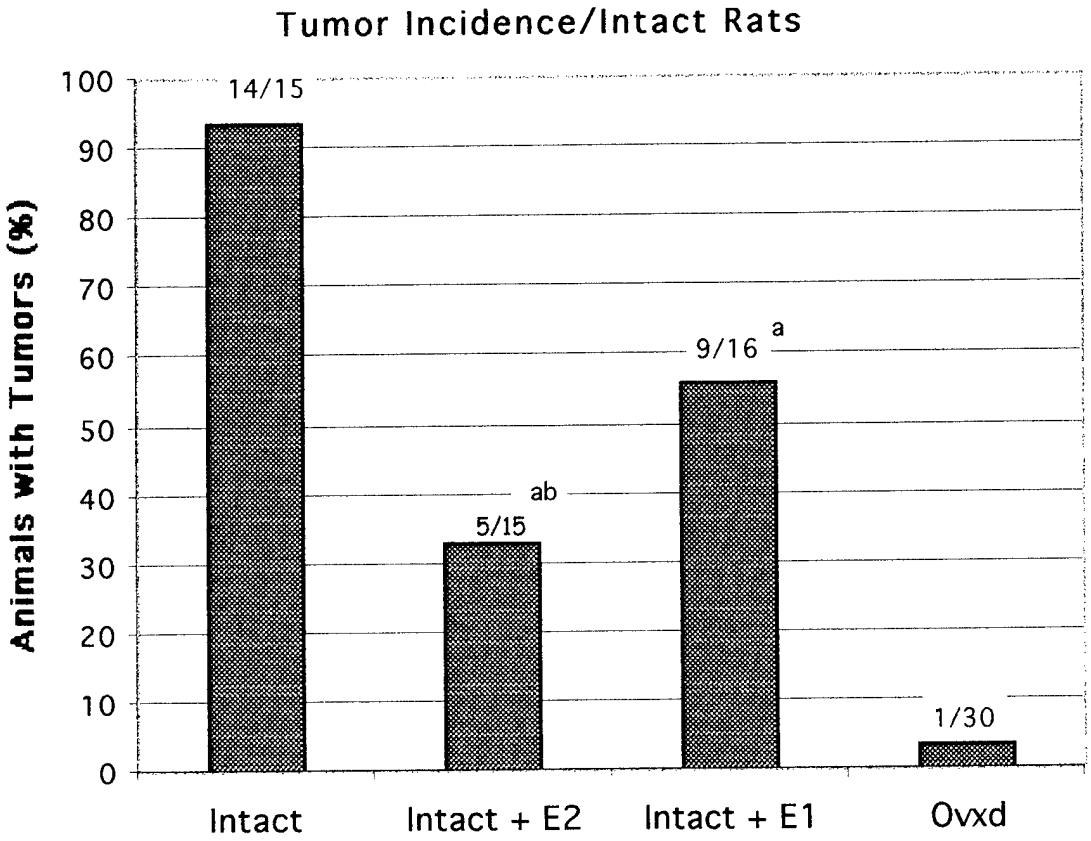

Figure 3. Tumor Incidence in MNU-Treated, Ovary-Intact Rats. Rats were injected with MNU and a Silastic capsule containing either estradiol (E2) or estrone (E1) was inserted subcutaneously. Ovary intact or ovariectomized (ovxd) rats served as controls for comparisons. a, $p < 0.05$ vs. ovxd; b, $p < 0.05$ vs. intact.

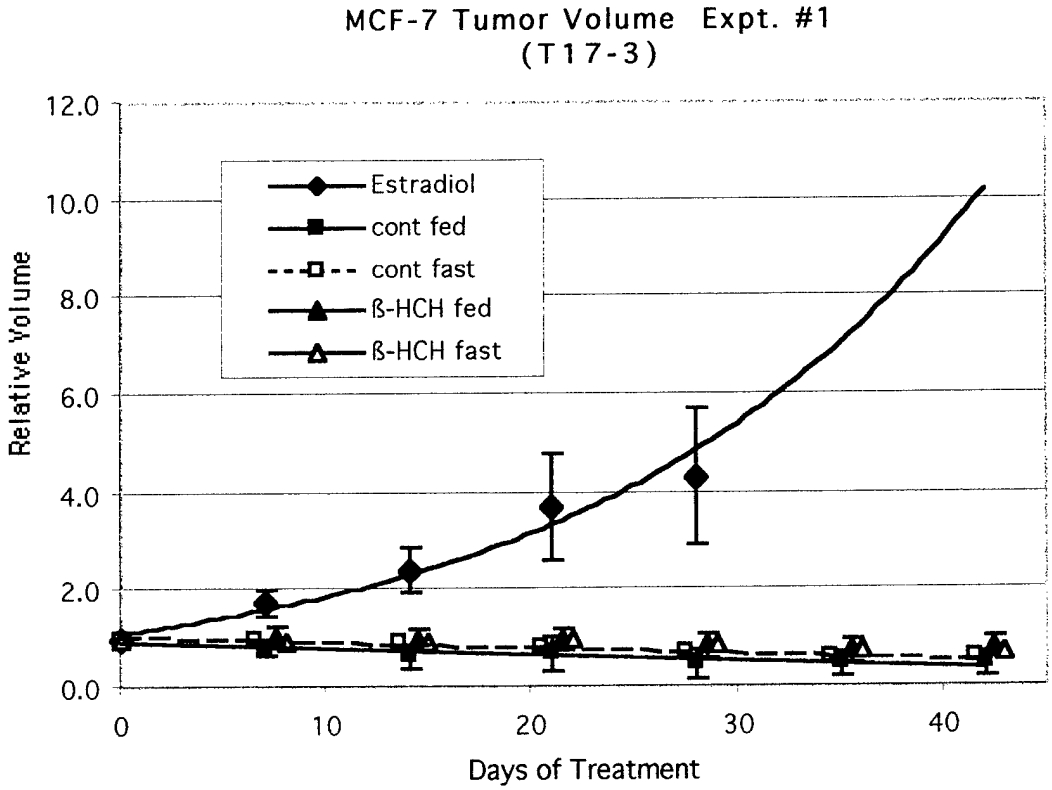

Figure 4. Effect of ß-HCH on MCF-7 Tumor Growth in Athymic Mice on Dietary Restriction. MCF-& tujor was transplanted to mice that were under estrogen stimulation via a subcutaneous capsule of estradiol. After 3 weeks the estradiol capsule was replaced with an empty control capsule (cont) or one containing ß-HCH (ß-HCH). Half of these hosts were placed on periodic dietary restriciton (fast) while the others were left on food ad libitum (fed).

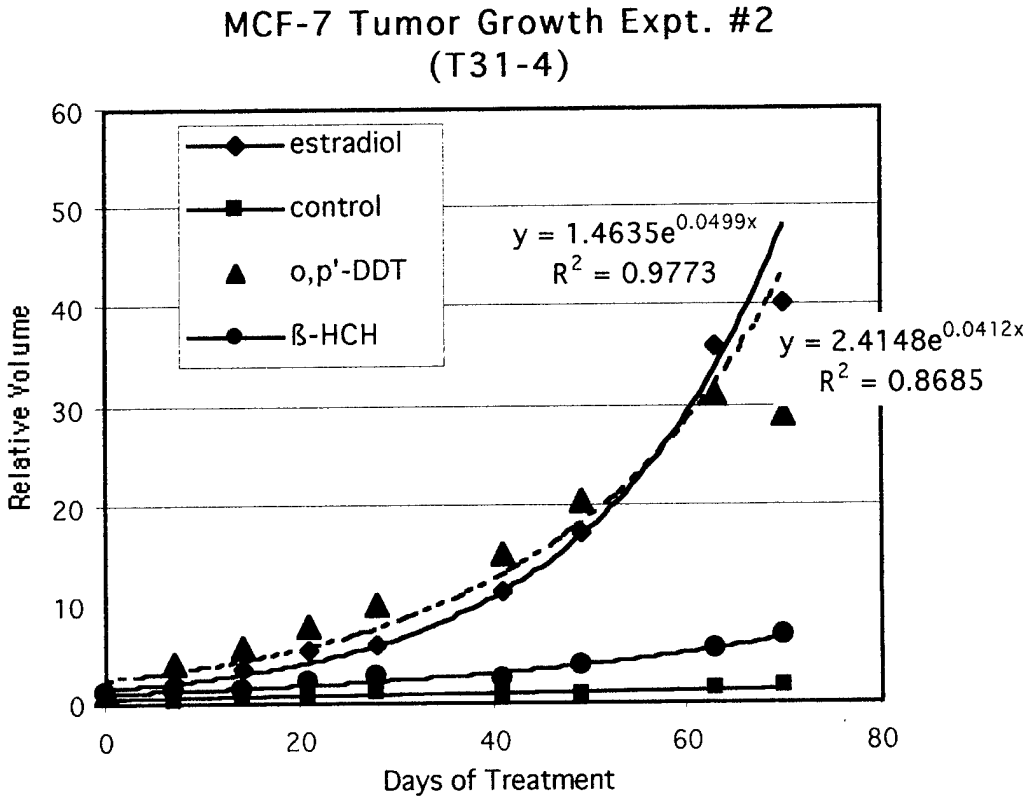

Figure 5. Effect of o,p'-DDT or ß-HCH on MCF-7 Tumor Growth. Tumor was transplanted from a host that had not been treated with estrogen to maintain the tumor's growth. This tumor was grown for 5 weeks in ovary intact animals with only 3 daily injections of estradiol administered at the time of transplantation.